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ORIGINAL ARTICLE

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What limits production of unusual monoenoic fatty acids in transgenic plants?

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Abstract Unusual monounsaturated fatty acids are major constituents (greater than 80%) in seeds of *Coriandrum sativum* L. (coriander) and *Thunbergia alata* Bojer, as well as in glandular trichomes (greater than 80% derived products) of *Pelargonium ×hortorum* (geranium). These diverged fatty acid structures are produced via distinct plastidial acyl-acyl carrier protein (ACP) desaturases. When expressed in *Arabidopsis thaliana* (L.) Heynh. under strong seed-specific promoters the unusual acyl-ACP desaturases resulted in accumulation of unusual monoene fatty acids at 1–15% of seed fatty acid mass. In this study, we have examined several factors that potentially limit higher production of unusual monoenes in transgenic oilseeds. (i) Immunoblots indicated that the introduced desaturases were expressed at levels equivalent to or higher than the endogenous Δ9 18:0-ACP desaturase. However, the level of unusual fatty acid produced in transgenic plants was not correlated with the level of desaturase expression. (ii) The unusual desaturases were expressed in several backgrounds, including antisense 18:0-ACP desaturase plants, in *fab1* mutants, and co-expressed with specialized ACP or ferredoxin isoforms. None of these experiments led to high production of expected products. (iii) No evidence was found for degradation of the unusual fatty acids during seed development. (iv) Petroselinic acid added to developing seeds was incorporated into triacylglycerol as readily as oleic acid, suggesting no major barriers to its metabolism by enzymes of glycerolipid assembly. (v) In vitro and in situ assay of acyl-ACP desaturases revealed a large discrepancy of activity when comparing unusual acyl-ACP desaturases with the endogenous Δ9 18:0-ACP desaturase. The combined results, coupled with the sensitivity of acyl-ACP desaturase activity to centrifugation and low salt or detergent suggests low production of unusual monoenes in transgenic plants may be due to the lack of, or incorrect assemble of, a necessary multi-component enzyme association.

Keywords *Coriandrum* · Monoene · Multi-component enzyme association · *Pelargonium* · Petroselinic acid · *Thunbergia*

Abbreviations ACP: acyl carrier protein · DAF: days after flowering · FAME: fatty acid methyl ester · FAS: fatty acid synthesis · Fd: ferredoxin · PUFA: polyunsaturated fatty acids · TAG: triacylglycerol · VLCFA: very long chain fatty acid

Introduction

Most plant seeds produce storage oils which consist predominantly of only a few fatty acids (palmitic, oleic, linoleic, and linolenic). However, within the plant kingdom, species occur that are capable of producing a diverse assortment of fatty acids (in excess of 200) with alterations in chain length, double-bond position and oxygenated functional groups (Kishore and Somerville 1993; van de Loo et al. 1993). For example, seed endosperm of *Coriandrum sativum* (coriander) and *Thunbergia alata* as well as glandular trichomes of *Pelargonium ×hortorum* (garden geranium) produce unusual monoenoic acids by action of Δ4 16:0-ACP, Δ6 16:0-ACP, and Δ9 14:0-ACP desaturases, respectively (Cahoon et al. 1992, 1994; Schultz et al. 1996). The products of these enzymes ($18{:}1^{\Delta 6}$ in coriander, $16{:}1^{\Delta 6}$ in *T. alata*, and $16{:}1^{\Delta 11}$, $18{:}1^{\Delta 13}$ and derived products in geranium) constitute over 80% of total fatty acids in these species.

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Some of these unusual fatty acids have useful chemical or physical properties that are potentially valuable for industrial commodities or as edible products (Ohlrogge 1994; Topfer et al. 1995). Most unusual fatty acids are produced by species not adapted for agriculture production and therefore utilization of this rich renewable resource is currently limited. However, if available at the current low cost of commodity vegetable oils, these unusual fatty acid structures might provide alternative industrial feedstocks that could serve as petrochemical replacements.

To achieve large-scale and low-cost production of specialty fatty acids, several research groups have isolated genes involved in unusual fatty acid biosynthesis and produced transgenic plants expressing these genes. However, in most cases the production level of unusual fatty acid structures in transgenic plants has been substantially below the level found in the species from which genes for its biosynthesis were derived (Cahoon et al. 1992, 2000; Broun and Somerville 1997; Lee et al. 1998; Schultz and Ohlrogge 2002). The factors that limit accumulation of high quantities of the targeted fatty acid structure in transgenic crops are not well understood. Future development of oilseed crops as sources of renewable industrial feedstocks may require better fundamental understanding of aspects of lipid metabolism that limit accumulation of specialty fatty acids. As part of this effort we have investigated the production of unusual monoenes in transgenic *Arabidopsis* that express three distinct acyl-ACP desaturases from coriander, *T. alata* or geranium. In this study we have investigated the influence of enzyme expression level, substrate availability, β-oxidation, and discrimination by lipid metabolism in transgenic *Arabidopsis* lines and we have characterized petroselinic acid biosynthesis in coriander extracts. Although no single factor was identified as limiting unusual monoene products in transgenic plants, our evidence suggests that accumulation of unusual monoenoic fatty acids may be limited by the lack of, or improper formation of, a functional multi-component enzyme association.

Materials and methods

Plant materials

Arabidopsis thaliana (L.) Heynh. (Columbia ecotype) was grown under 16 h of light (90 µmol photons m^{-2} s^{-1}; 22 °C) and 8 h of darkness (20 °C). The *Arabidopsis fab1* mutant line (James and Dooner 1990), which is defective in β-ketoacyl-ACP synthase (KAS) II activity (Wu et al. 1994) was obtained from John Browse. *Coriandrum sativum* L. (coriander) was cultivated in a greenhouse with no supplemental lighting at 21–25 °C (East Lansing, Mich.). Developing seeds from coriander were harvested; the endosperm was dissected from the pericarp and seed coat of mericarps, and was immediately used in enzyme assays.

Binary-vector construction and *Arabidopsis* transformation

The binary vectors containing the Δ4 or Δ6 16:0-ACP desaturase under control of the napin promoter were provided by Edgar Cahoon. The binary vector containing antisense-oriented Δ9 18:0-ACP desaturase was a generous gift from Jean Kridl. To ensure proper plastid targeting, the coding region for the Δ9 14:0-ACP desaturase and the *Anabaena* sp. 7120 ferredoxin (Fd) were fused in-frame with the coriander Δ4 16:0-ACP desaturase transit peptide and subsequently cloned under control of the napin promoter in pRD410 derived from pBin19 (Datla et al. 1992). The coriander seed-specific ACP (Suh et al. 1999) was cloned under control of the USP promoter in pBin-USP-Hyg derived from pBin19 (Bevan 1984; Baumlein et al. 1991). All recombinant binary vectors were transformed into *Agrobacterium tumefaciens* strain C58C1 by the direct-uptake method (An 1987).

Wild-type *Arabidopsis* was grown on mounded soil covered with nylon screen until primary bolts were approximately 10 cm, then transformed by vacuum-infiltration based on the procedures of Bechtold et al. (1993) and Bent et al. (1994). Recombinant *A. tumefaciens* was grown to $O.D_{600} > 2.0$ in 0.5 l YEP medium (10 g/l bacto peptone, 10 g/l yeast extract, and 5 g/l NaCl) supplemented with 50 mg/l rifampicin, 25 mg/l gentamicin, and 50 mg/l kanamycin. Cells were collected by centrifugation (3,000 *g*, 15 min, 4 °C) and resuspended in 1 l infiltration medium [2.2 g Murashige and Skoog (MS) salts, 1×B5 vitamins, 50 g sucrose, 0.5 g Mes; pH to 5.7 with KOH, supplemented with 4.4×10^{-8} M benzylaminopurine and 0.2 ml Silwet L-77]. Co-transformations were made using equal volumes of two recombinant *A. tumefaciens* suspensions. To transform *Arabidopsis*, the plants were immersed in the *A. tumefaciens* suspension, and then placed under vacuum (58 kPa) until leaf tissue appeared uniformly water-soaked (approx. 5 min). Following vacuum-infiltration, plants were grown as described above until seeds were fully matured. Seeds (T_1) were bulk-harvested from each pot, sterilized in 50% bleach containing 0.02% Triton X-100 for 7 min, rinsed several times in distilled H_2O, resuspended in 3 ml 0.1% agarose in distilled H_2O, then dispensed and germinated on selective medium (4.3 g/l MS salts, 1×B5 vitamins, 1% sucrose, 0.5 g/l Mes, and 0.8% phytagar) containing 100 mg/l carbenicillin and 50 mg/l kanamycin and/or 20 mg/l hygromycin. Seeds (T_1) obtained from co-transformed *Arabidopsis* were selected on media supplemented with both kanamycin and hygromycin. Seedlings resistant to selection antibiotic were transferred to soil for production of T_2 seed.

Western blot analysis of transgenic plants

Developing seeds or siliques of *Arabidopsis* were homogenized in 1× SDS sample loading buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% glycerol, 0.1 M DTT, and 0.025% bromophenol blue] and incubated at 95 °C for 5 min. Samples were centrifuged at 15,000 *g* for 5 min and aliquots of the supernatant were electrophoresed on 11% (for desaturase) or 15% (for ACP) SDS-PAGE gels according to the method of Laemmli (1970). Proteins were transferred to nitrocellulose membrane and probed as described by Harlow and Lane (1988) using antisera against avocado Δ9 18:0-ACP desaturase, *Thunbergia alata* Bojer Δ6 16:0-ACP desaturase, or spinach ACP-I.

Fatty acid composition

Prior to derivatization, heptadecanoic acid (17:0) was added to each sample as an internal standard. Each sample was trans-methylated at 90 °C for 1 h in 0.5 ml toluene and 1 ml 1 N methanolic HCl. Following trans-methylation, 1 ml of aqueous 0.9% NaCl was added, and fatty acid methyl esters (FAMEs) were recovered by three sequential extractions with 1 ml of hexane. Alternatively, to achieve base-line separation of $18{:}1^{\Delta6}$ and $18{:}1^{\Delta9}$ some samples were trans-isopropylated as described by Wolff and co-workers (Wolff and Fabien 1989; Wolff and Vandamme 1992). Fatty acid esters were analyzed by gas chromatography using a CP-Sil88 column (50 m long, 0.25 mm i.d.; Chrompack), an oven temperature programmed from 150 °C to 200 °C at 2 °C min^{-1}, and a column head pressure of 150 kPa He. To better facilitate separation of $18{:}1^{\Delta6}$ and $18{:}1^{\Delta9}$, oven temperature programming was changed to 150 °C to 200 °C at 0.8 °C/min. The identity of

fatty acids was determined by comparing retention times and mass spectra with standards.

In vitro synthesis of $18:1^{\Delta 6}$

The synthesis of $18:1^{\Delta 6}$ acid from [1-^{14}C]malonyl-CoA was conducted according to the method of Cahoon and Ohlrogge (1994b). [1-^{14}C]malonyl-CoA was synthesized using isolated pea chloroplast as described by Roughan (1994). Coriander endosperm was homogenized in extraction buffer [100 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2.5 mM DTT, 1 mM $MgCl_2$, 1 mM KCl, 1 mM isoascorbate, 0.1% BSA] and the homogenate (filtered with two layers of Miracloth) or the supernatant (after two centrifugations of the homogenate at 15,000 *g* for 10 min, 4 °C) was incubated in 0.25 ml assay buffer (2 mM ascorbate, 0.75 mM NADPH, 1 mM NADH [NADPH and NADH prepared fresh in 0.1 M Tricine, pH 8.0], 3,200 units/ml catalase, 2 mM ATP, 32 mM Pipes [pH 6.0], and 10.4×10^{-3} mM [1-^{14}C]malonyl-CoA [2 GBq/mmol]) at 25 °C for 20 min with shaking at 100 rpm. The reaction was terminated by addition of 40 µl of glacial acetic acid and 4.5 ml of acetone, and the reaction was completely dried under N_2 gas. Reaction products were subsequently methyl-esterified (as described above) and FAMEs were analyzed by argentation TLC as described by Morris et al. (1967). After detection by autoradiography, radiolabeled FAMEs were scraped from TLC plates and quantitated by liquid scintillation counting.

Incorporation of fatty acids into triacylglycerol (TAG)

Developing *Arabidopsis* seeds (wild type) were harvested 10 days after flowering. A small portion of the seed coat was torn away using fine forceps under a stereomicroscope (10×). Seeds were immediately placed into a glass incubation vial containing 1 ml 0.1 M phosphate buffer (pH 7.0). Incubations containing at least 200 seeds per sample were started by addition of 0.2 mM [^{3}H]$18:1^{\Delta 6}$ (89 MBq/mmol) and [^{14}C]$18:1^{\Delta 9}$ (89 MBq/mmol) as a dual-label mixture. Fatty acid substrates were supplied as ammonium salts in 80% ethanol, with final ethanol concentration in each reaction below 2.5%. Seeds were incubated with the dual-substrate mixture at room temperature for 4 h with shaking at 150 rpm. Reactions were terminated by rapidly removing the labeling buffer and rinsing three times with 0.1 M phosphate buffer (pH 7.0). Samples were immediately ground using a glass homogenizer, and lipids were recovered as described by Bligh and Dyer (1959). The chloroform fraction containing the labeled lipids was evaporated to dryness under a steady stream of N_2 then re-dissolved in 0.1 ml hexane. Lipid classes were separated by TLC (K6 silica plates; Whatman, Clifton, N.J., USA) using two developments (to 6 and 12 cm) in chloroform/methanol/acetic acid (75:25:8, by vol.) and a third development (to 18 cm) in hexane/ethyl ether/acetic acid (60:40:1; by vol.). Plates were dried completely between developments. Lipids were identified by a light I_2 staining and comparison with known standards. Radiolabeled lipids (^{14}C) were identified using an Instant Imager (Packard Instruments), then scraped from the plates and the composition of ^{3}H and ^{14}C was determined using a scintillation counter.

In vitro acyl-ACP desaturase activities in transformed *Arabidopsis* lines

To assess the activity of the introduced unusual acyl-ACP desaturase relative to the endogenous Δ9 18:0-ACP desaturase, in vitro acyl-ACP desaturase assays were performed on *Arabidopsis* lines transformed with the *T. alata* Δ6 16:0-ACP desaturase or the geranium Δ9 14:0-ACP desaturase. Developing seeds [8–10 days after flowering (DAF)] were harvested from at least 10 siliques, and placed in 0.75 ml extraction buffer [0.1 M Tris-HCl (pH 7.6), 1 mM EDTA, 5 mM DTT, 1 mM $MgCl_2$, 1 mM KCl, 1 mM ascorbate, 0.01% BSA, 1% PVP, 2% PVPP] on ice. Seeds were homogenized using a plastic micro-centrifuge pestle. Insoluble material was pelleted by two centrifugations (13,000 *g*, 5 min, 4 °C). Protein content was estimated from the supernatant using the Bradford (1976) assay. [1-^{14}C]Acyl-ACP substrates were prepared using the procedure of Rock and Garwin (1979) with ACP from *Escherichia coli*. All labeled fatty acids used for the synthesis of acyl-ACP had specific activities of 2.1 MBq/mmol (American Radiolabel Company, St. Louis, Mo., USA). Acyl-ACP desaturase assays were conducted as previously reported (Cahoon et al. 1994) with the following modifications. Assays contained 0.03 mg protein extract, 0.2 mg/ml BSA, 10 µM *Impatiens balsamina* Fd (described in Schultz et al. 2000) instead of spinach Fd; maize root ferredoxin-NADP oxidoreductase (FNR) was used instead of spinach FNR, and 124 pmol substrate (14:0-ACP, 16:0-ACP, or 18:0-ACP) was supplied. Assays were incubated at room temperature (21 °C) with shaking (100 rpm) for noted times. Reactions were terminated and saponified as described by Cahoon et al. (1994). Fatty acids were methylated as described above.

Seed chloroplast isolation and labeling

Isolation and [1-^{14}C]acetate labeling of developing *Arabidopsis* seed plastids was adapted from the procedure of Kang and Rawsthorne (1994). Siliques ranging in age from 9 to 11 DAF were harvested from five tagged flower bolts. Developing seeds were immediately dissected from individual siliques, then placed on ice in 1 ml plastid isolation media (PIM) containing 0.5 M sorbitol, 20 mM Hepes (pH 7.4), 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA (pH 8.0), 5 mM DTT, and 1% BSA. Within 1 h of harvest, the seeds were homogenized in a hand held-glass homogenizer and the homogenate passed through two layers of Miracloth into a microcentrifuge tube. Plastids were pelleted by centrifugation at 500 *g*, 10 min, 4 °C, and then resuspended in a minimal volume of PIM. Chlorophyll content of the sample was determined as described by Arnon (1949). Plastid labeling buffer was identical to PIM with the addition of 10 mM $NaHCO_3$, 4 mM ATP, 0.5 mM NADH, 0.5 mM NADPH, 37 kBq [1-^{14}C]acetate, and 0.2 mM CoA. Reaction volume was 0.1 ml and reactions were started by addition of 0.03 mg chlorophyll equivalent plastid preparation. Reactions were incubated at room temperature with 150 rpm shaking for 40 min and were terminated by addition of 0.85 ml 2.35 N NaOH. Samples were saponified by heating to 95 °C for 1 h, then cooled to room temperature and acidified to <3.0 by addition of 4 M H_2SO_4. Carrier fatty acid (0.04 mg) was added and samples were extracted three times with 2 ml hexane. After concentration under N_2, samples were methylated and analyzed by argentation TLC as described above.

Results

Analysis of monoene composition in relation to desaturase protein levels

In a previous study, expression of the coriander Δ4 16:0-ACP desaturase under control of the strong constitutive 35S promoter resulted in $16:1^{\Delta 4}$ and $18:1^{\Delta 6}$ accumulation up to approximately 10% of total fatty acids in transgenic tobacco calli (Cahoon et al. 1992). Lower levels were detected in leaves and seeds of plants regenerated from these calli. To further investigate the influence of tissue type and expression level on production of unusual monoenoic acids, the specialized acyl-ACP desaturases were cloned under control of the strong seed-specific napin promoter and transformed into *Arabidopsis*. Production of $16:1^{\Delta 4}$ and $18:1^{\Delta 6}$ ranged from 0.5 to 1% for lines transformed with the coriander Δ4 16:0-ACP desaturase. In plants transformed with the

T. alata Δ6 16:0-ACP desaturase, $16{:}1^{\Delta 6}$ was not detected but the elongation products $18{:}1^{\Delta 8}$ and $20{:}1^{\Delta 10}$ were identified at combined levels of approximately 4% of total fatty acids. *Arabidopsis* transformed with the geranium Δ9 14:0-ACP desaturase yielded no immediate reaction product ($14{:}1^{\Delta 9}$) but the elongation products ($16{:}1^{\Delta 11}$, $18{:}1^{\Delta 13}$ and $20{:}1^{\Delta 15}$) ranged from trace amounts to 13% with most lines near 7%. Thus, the products of these three different unusual acyl-ACP desaturases remained a minor component of the seed oil despite the use of a strong seed promoter.

To investigate whether monoene production was limited by low desaturase expression, the levels of Δ4 16:0-ACP desaturase and Δ6 16:0-ACP desaturase in transgenic plants were estimated by western blot analysis of siliques harvested at 9–12 DAF and using antibodies raised against avocado Δ9 18:0-ACP desaturase or *T. alata* Δ6 16:0-ACP desaturase, respectively. As shown in Fig. 1a, western blot analysis of *Arabidopsis* lines transformed with the Δ4 16:0-ACP desaturase displayed two strong bands at 37 kDa and 36 kDa. The 37-kDa band was detected in both wild-type and transgenic *Arabidopsis* and thus likely represents the ubiquitous Δ9 18:0-ACP desaturase. The 36-kDa band was observed only in transformed lines and co-migrated with the Δ4 16:0-ACP desaturase in protein extracts of coriander seed endosperm. Production of unusual monoenes was determined in mature seeds of each line (indicated below each lane, see Fig. 1a). Similarly, western blot analysis of the *T. alata* Δ6 16:0-ACP desaturase in *Arabidopsis* transformants revealed a band at approximately 38 kDa which co-migrated on SDS-PAGE with the Δ6 16:0-ACP desaturase of *T. alata* extracts (Fig. 1b). Only a weak band at 37 kDa, corresponding to the Δ9 18:0-ACP desaturase, was detected in the wild type and in transformant lines. As expected from position effects associated with plant transformations, expression of the acyl-ACP desaturases varied substantially between independent transformants, but two observations were consistent between plants transformed with either desaturase. First, based on western blot signals, levels of the introduced novel acyl-ACP desaturases were at least equal to that of the endogenous 18:0-ACP desaturase, and in some cases clearly higher. Second, accumulation of the unusual monoenes did not correlate with desaturase expression levels.

Are unusual monoenes degraded during seed development?

Low levels of unusual monoene accumulation could result from limited biosynthesis or from degradation of the unusual product after its synthesis. Transient accumulation or later losses of unusual fatty acids would indicate possible product degradation due to *β*-oxidation. Therefore, to assess possible losses of unusual monoenes due to *β*-oxidation, we examined the developmental profile of lipid deposition in lines expressing unusual acyl-ACP desaturases. The fatty acid compositions of developing seeds from Δ4 16:0-ACP-transformed lines and Δ9 14:0-ACP-desaturase-transformed lines were analyzed at several stages throughout seed development (Fig. 2). For both transgenic lines, accumulation of unusual monoenes ($16{:}1^{\Delta 6}$ and $18{:}1^{\Delta 8}$, Fig. 2a; $14{:}1^{\Delta 9}$, $16{:}1^{\Delta 11}$, and $18{:}1^{\Delta 13}$, Fig. 2b) was similar to the accumulation of endogenous monoenes ($16{:}1^{\Delta 9}$, $18{:}1^{\Delta 9}$, and $18{:}1^{\Delta 11}$). Thus we could find no evidence of a transient accumulation of unusual monoenes that might suggest their breakdown. In addition, in preliminary experiments, we could detect no increase in acyl-CoA oxidase activity in tissues expressing the coriander desaturase (data not shown).

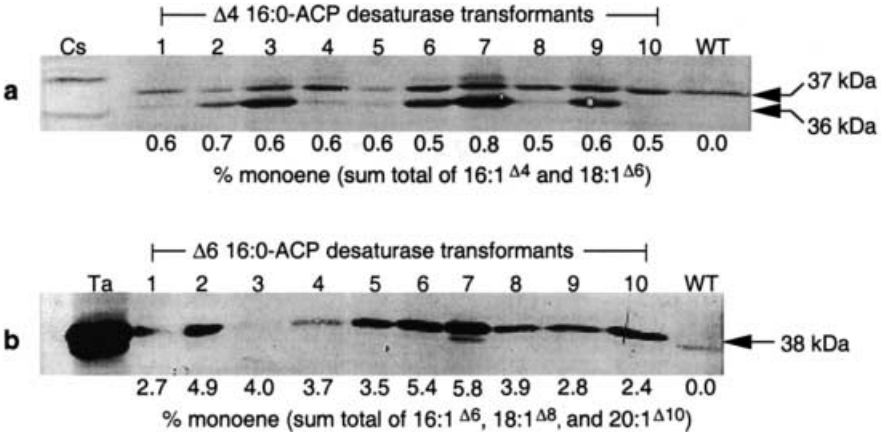

Fig. 1. Analysis of unusual monoene accumulation and expression of the Δ4 16:0-ACP desaturase (**a**) and the Δ6 16:0-ACP desaturase (**b**) in transgenic *Arabidopsis thaliana*. Total proteins (0.1 mg) were isolated from siliques of the wild type (*WT*) and *Arabidopsis* lines transformed with either the Δ4 16:0-ACP (**a**, *1–10*) or the Δ6 16:0-ACP desaturase (**b**, *1–10*) and probed with antisera against avocado Δ9 18:0-ACP desaturase (**a**) or the *Thunbergia alata* Δ6 16:0-ACP desaturase (**b**). Total fatty acids from mature T_2 seeds were analyzed by GC, and unusual monoene composition is shown for each individual transformant line at the bottom of the western blot. The % unusual monoenes are the combined amounts of $16{:}1^{\Delta 4}$ and $18{:}1^{\Delta 6}$ (**a**, *1–10*) or the combined amount of $16{:}1^{\Delta 6}$, $18{:}1^{\Delta 8}$, and $20{:}1^{\Delta 10}$ (**b**, *1–10*). Total protein extracts (30 μg) from endosperm of coriander (*Coriandrum sativum*; *Cs*), and *T. alata* (*Ta*) were used as standards

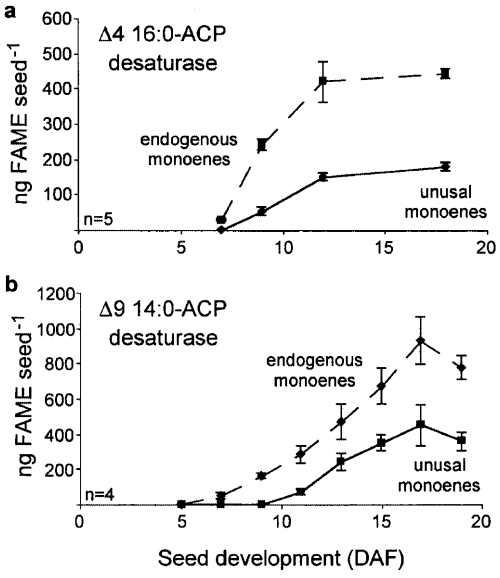

Fig. 2a, b. Analysis of monoene accumulation in *Arabidopsis* expressing unusual acyl-ACP desaturases. Production of unusual monoenes ($16{:}1^{\Delta4}$ and $18{:}1^{\Delta6}$) in *Arabidopsis* lines transformed with the Δ4 16:0-ACP desaturase (**a**) and unusual monoenes ($14{:}1^{\Delta9}$, $16{:}1^{\Delta11}$, $18{:}1^{\Delta13}$) in *Arabidopsis* lines transformed with the Δ9 14:0-ACP desaturase (**b**) were compared with the endogenous monoenes ($16{:}1^{\Delta9}$, $18{:}1^{\Delta9}$ and $18{:}1^{\Delta11}$). Fatty acid composition of seeds was analyzed between 5 and 20 DAF in independent transformation lines (*Arabidopsis fab1* transformed with the Δ4 16:0-ACP desaturase, $n=5$; *Arabidopsis* wild type transformed with the Δ9 14:0-ACP desaturase, $n=4$). Data are means ± SE

Incorporation of unusual monoenes into storage lipids

The biosynthesis of TAG with unusual monoenes in both *T. alata* and coriander has been found to exhibit a transient flux of monoenes through phosphatidylcholine prior to incorporation into TAG (Cahoon and Ohlrogge 1994a; Schultz and Ohlrogge 2000). Several enzymes in the storage-lipid biosynthetic pathway, such as acyltransferases, may provide specificity that could prevent utilization of unusual monoenes and thus limit monoene accumulation (Dutta et al. 1992). To investigate incorporation of unusual monoene lipids into TAG, we conducted a dual-labeling competition assay by incubating developing *Arabidopsis* seeds with $[^3H]18{:}1^{\Delta6}$ and $[^{14}C]18{:}1^{\Delta9}$. As shown in Table 1, the rate of $18{:}1^{\Delta6}$ incorporation into TAG was not distinguishable from that of $18{:}1^{\Delta9}$. Furthermore, comparison of the ratios for $[^3H]18{:}1^{\Delta6}$ and $[^{14}C]18{:}1^{\Delta9}$ supplied as substrate (1.3 ± 0.3, $n=3$) compared with the ratios for $[^3H]18{:}1^{\Delta6}$ and $[^{14}C]18{:}1^{\Delta9}$ incorporated into TAG (0.9 ± 0.1, $n=3$) indicate both $[^3H]18{:}1^{\Delta6}$ and $[^{14}C]18{:}1^{\Delta9}$ were equally utilized as substrates for TAG synthesis.

Table 1. Dual-isotope labeling of developing *Arabidopsis thaliana* seed TAG with $[^3H]18{:}1^{\Delta6}$ and [14 C]$18{:}1^{\Delta9}$. Fatty acid incorporation (pmol/seed) values represent the average ± SE of three replicates

Sample	Incorporation h^{-1}	
	pmol $[^3H]18{:}1^{\Delta6}$ $seed^{-1}$	pmol $[^{14}C]18{:}1^{\Delta9}$ $seed^{-1}$
TAG	0.82 ± 0.5	0.71 ± 0.5

Influence of substrate level on the production of unusual monoenes

The substrate for the unusual acyl-ACP desaturases in coriander and *T. alata* is 16:0-ACP rather than the 18:0-ACP which is used by the ubiquitous Δ9 18:0-ACP desaturase. To investigate the influence of acyl-ACP substrate level on the production of unusual monoenoic acids in transgenic plants, Δ4 16:0-ACP and Δ6 16:0-ACP desaturases were transformed into *Arabidopsis fab1*. This mutant line is deficient in elongation of 16:0-ACP to 18:0-ACP (Wu et al. 1994) and thus seeds have an approximately 2-fold increase in 16:0 and a several-fold increase in $16{:}1^{\Delta9}$. Although 16:0-ACP pools have not been quantified in the *fab1* mutant, an increased level of 16:0-ACP can be inferred from the increased level of $16{:}1^{\Delta9}$ because the Δ9 18:0-ACP desaturase, although less active on 16:0-ACP, has a similar K_m value for 16:0-ACP and 18:0-ACP (Gibson 1993). Furthermore, Cahoon and Shanklin (2000) found an approximately 2-fold increase in unusual monoenes when a variant acyl-ACP desaturase (with increased 16:0-ACP specificity) was expressed in *fab*1 compared with the wild type.

Wild-type and *fab1 Arabidopsis* were transformed with the Δ4 16:0-ACP or Δ6 16:0-ACP desaturase and the resulting seed (T_2) fatty acid composition was analyzed (Table 2). Wild-type lines transformed with the

Table 2. Fatty acid profiles of seeds from transgenic *Arabidopsis*, wild type or *fab1*, expressing the Δ4 16:0-ACP desaturase or the Δ6 16:0-ACP desaturase

Genotype[b]	Fatty acid (mol%)			
	Unusual monoenes[c]	Saturates[d]	PUFA + VLCFA[e]	Monoenes[f]
Wild type ($n=2$)	0.0	12.8	70.4	16.8
fab1 ($n=2$)	0.0	22.5	64.9	12.6
4DE/wild type ($n=10$)	0.6 ± 0.04	13.3 ± 0.2	73.6 ± 0.6	12.5 ± 0.4
4DE/*fab1* ($n=6$)	2.4 ± 0.2	26.7 ± 0.6	62.8 ± 0.6	9.3 ± 0.4
6DE/wild type ($n=10$)	4.0 ± 0.3	13.7 ± 0.2	68.1 ± 0.6	14.3 ± 0.4
6DE/*fab1* ($n=6$)	3.2 ± 0.2	22.3 ± 0.6	64.1 ± 0.3	12.0 ± 0.2

[a]Fatty acid profiles of individual genotypes were determined by GC analysis of *n* replicates. Mean values ± SE
[b]Wild type or *fab1* lines were transformed with the Δ4 16:0-ACP desaturase (4DE) or with the Δ6 16:0-ACP desaturase (6DE)
[c]The sum composition of $16{:}1^{\Delta4}$ and $18{:}1^{\Delta6}$ in 4DE lines and the sum composition of $16{:}1^{\Delta6}$, $18{:}1^{\Delta8}$ and $20{:}1^{\Delta10}$ in 6DE lines
[d]The sum composition of 16:0, 18:0, and 20:0 fatty acids
[e]Polyunsaturated fatty acids plus very long-chain fatty acids: the sum composition of 18:2, 18:3, and $20{:}1^{\Delta11}$
[f]The sum composition of $16{:}1^{\Delta9}$ and $18{:}1^{\Delta9}$

Δ4 16:0-ACP desaturase contained an average unusual fatty acids ($16:1^{\Delta4}$ and $18:1^{\Delta6}$) content of less than 1% in the wild-type background but nearly 3% in the *fab*1 background. The ratio of $16:1^{\Delta4}$ and $18:1^{\Delta6}$ (approx. 2:1) was similar in both genetic backgrounds (wild type and *fab1*). In contrast to the results with the Δ4 16:0-ACP desaturase, expression of the Δ6 16:0-ACP desaturase in the *fab1* background did not lead to increased production of unusual monoenes relative to the wild-type background (Table 2).

Can unusual monoene production be increased by reducing competition for substrates and co-factors?

A possible explanation for the ineffectiveness of the unusual acyl-ACP desaturases in heterologous systems might be due to competition with the native Δ9 18:0-ACP desaturase for endogenous substrates and co-factors. To increase accessibility of the introduced acyl-ACP desaturases to substrates and co-factors, the expression level of Δ9 18:0-ACP desaturase was reduced by antisense gene constructs.

To isolate Δ9 18:0-ACP desaturase antisense lines, fatty acid profiles were analyzed from more than 100 independent transformant lines. One high-stearate (18:0) transgenic line (Anti9DE-103) with an approximately 4-fold increase in 18:0 (12%) compared with the wild type (3%) was identified. The increased level of 18:0 was accompanied by enhanced accumulation of long-chain saturated fatty acids (20:0, 22:0, and 24:0, data not shown). *Arabidopsis* line Anti9DE-103 was crossed with lines expressing the Δ4 16:0-ACP desaturase (4DE lines) or with lines expressing the Δ6 16:0-ACP desaturase (6DE lines). Monoene composition of bulked F_2 seed (from a single F_1 plant) was analyzed and compared with parental genotypes (Table 3). No appreciable increase in unusual monoene composition was detected in the Anti9DE-background.

Influence of ACP and Fd isoforms

Specific isoforms of ACP and Fd have recently been found to influence acyl-ACP desaturase activity in vitro (Suh et al. 1999; Schultz et al. 2000). A seed-specific coriander ACP supported 4- and 10-fold higher Δ4 16:0-ACP desaturase activity than spinach ACP or *E. coli* ACP, respectively. In addition, the source of Fd (heterotrophic or photosynthetic) was found to influence acyl-ACP desaturase activity by greater than 4-fold. To enhance monoene production, a coriander ACP and *Anabaena* Fd were co-expressed with unusual acyl-ACP desaturases. Double transformants expressing the coriander seed-specific ACP-1 with the Δ4 16:0-ACP desaturase were established by co-transformation with mixed cultures of *A. tumefaciens* carrying a coriander ACP-I gene or a Δ4 16:0-ACP desaturase gene. Lines expressing both the geranium Δ9 14:0-ACP desaturase and the *Anabaena* Fd were developed by reciprocal cross-pollination of single-loci homozygous lines.

Co-expression of the coriander Δ4 16:0-ACP desaturase and coriander ACP-I was assessed by western blot analysis using antisera to avocado Δ9 18:0-ACP desaturase (Fig. 3a) and spinach ACP-I (Fig. 3b). Expression of the Δ4 16:0-ACP desaturase was detected at 36 kDa while expression of the coriander ACP-1 was detected by bands at 15 kDa and 14 kDa. The 15-kDa signal corresponded with coriander ACP-I mature protein expressed in *E. coli* (data not shown). Overexpression of ACP in heterologous systems results in production of both holo- and apo-ACPs (Post-Beittenmiller et al. 1989). Thus, the 14-kDa signal likely corresponds to the coriander apo ACP-I (without prosthetic group). Expression of the coriander ACP-I had no influence on

Table 3. Fatty acid profiles of seeds from *Arabidopsis* transgenic lines expressing the Δ4 16:0-ACP desaturase or the Δ6 16:0-ACP desaturase in wild-type or antisense Δ9 18:0-ACP desaturase backgrounds

Genotype[b]	Fatty acid (mol%)			
	Unusual monoenes[c]	Saturates[d]	PUFA + VLCFA[e]	Monoenes[f]
Wild type ($n=2$)	0.0	13.4	70.2	16.5
Anti9DE ($n=1$)	0.0	28.3	62.9	8.8
4DE/wild type ($n=2$)	0.6	12.6	74.9	11.8
4DE/Anti9DE ($n=6$)	0.2 ± 0.04	25.1 ± 1.7	65.8 ± 1.3	8.9 ± 0.4
6DE/wild type ($n=4$)	4.1 ± 0.8	13.3 ± 0.1	68.7 ± 0.5	13.9 ± 0.3
6DE/Anti9DE ($n=5$)	1.8 ± 0.1	22.0 ± 1.0	64.0 ± 0.7	12.2 ± 0.4

[a]Fatty acid profiles of individual genotypes were determined by GC analysis of *n* replicates. Mean values ± SE
[b]Wild type or homozygous antisense Δ9 18:0-ACP desaturase lines were transformed with the Δ4 16:0-ACP desaturase (4DE) or with the Δ6 16:0-ACP desaturase (6DE). F_2 seed were analyzed
[c]The sum composition of $16:1^{\Delta4}$ and $18:1^{\Delta6}$ in 4DE lines and the sum composition of $16:1^{\Delta6}$, $18:1^{\Delta8}$ and $20:1^{\Delta10}$ in 6DE lines
[d]The sum composition of 16:0, 18:0, and 20:0 fatty acids
[e]The sum composition of 18:2, 18:3, and $20:1^{\Delta11}$
[f]The sum composition of $16:1^{\Delta9}$ and $18:1^{\Delta9}$

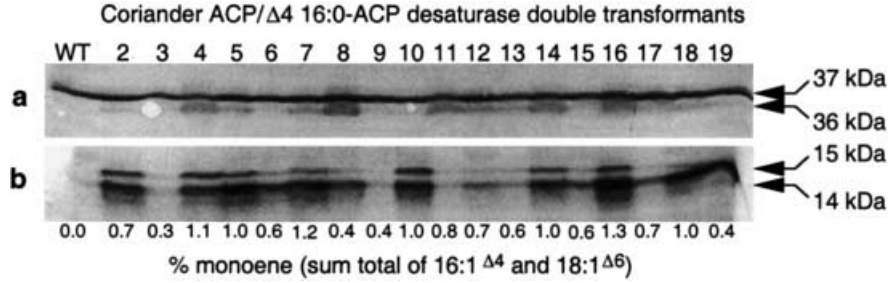

Fig. 3. Analysis of unusual monoene accumulation in *Arabidopsis* lines expressing the Δ4 16:0-ACP desaturase (**a**) and the coriander seed-specific ACP-I (**b**). Total proteins (100 μg) isolated from seeds of the wild type (*WT*) and double transformant lines (*lanes 2–19*) were probed with antisera against avocado Δ9 18:0-ACP desaturase (**a**) or spinach ACP (**b**), respectively. Estimated size (kDa) for detected proteins is noted in the margin. Total unusual monoene accumulation (% fatty acid composition of $16:1^{\Delta4}$ and $18:1^{\Delta6}$) is listed at the bottom of each lane

levels of endogenous ACPs (data not shown). Both coriander ACP-I and Δ4 16:0-ACP desaturase were highly expressed in transgenic lines #4, #14, and #16 (Fig. 3). However, accumulation of unusual monoenes was not appreciably influenced by co-expression of the desaturase with ACP, and no correlation was detected between coriander ACP-I and Δ4 16:0-ACP desaturase levels and monoene accumulation (Fig. 3). The fatty acid profiles from double transformant lines (Cs-ACP-I/Δ4 16:0-ACP desaturase) were compared with those of the wild type and single transformant lines of the Δ4 16:0-ACP desaturase (Table 4). In both the Cs-ACP-I/Δ4 16:0-ACP desaturase lines and the Δ4 16:0-ACP desaturase lines, production of $16:1^{\Delta4}$ and $18:1^{\Delta6}$ remained less than 1%.

To assess the influence of Fd on acyl-ACP desaturase activity, a homozygous *Arabidopsis* Δ9 14:0-ACP desaturase transformant line (9DE-14) was crossed reciprocally with two distinct homozygous *Arabidopsis* transformant lines expressing the *Anabaena* Fd (AnFd-1 and AnFd-2). Since bulked F_2 seed were analyzed for each cross, only 9/16 (56%) of the seed analyzed contained an allele for both the Δ9 14:0-ACP desaturase and *Anabaena* Fd while 12/16 (75%) of the seed analyzed contained an allele for the Δ9 14:0-ACP desaturase. Therefore the actual monoene composition of the F_2 seed may be slightly underestimated. To account for allelic influences on unusual monoene accumulation, we also analyzed the heterozygous sibling line (9DE-het) of 9DE-14. Co-expression of the Δ9 14:0-ACP desaturase with the *Anabaena* Fd did not result in appreciable increases in unusual monoene composition (Table 5). The small reduction in monoene composition in F_2 seeds relative to the parental line is likely due to the segregation of the Δ9 14:0-ACP desaturase in this population or an allelic effect.

Assay of unusual acyl-ACP desaturase activity in developing *Arabidopsis* seeds

Because western blots (Figs. 1, 3) indicated that the unusual acyl-ACP desaturase were highly expressed but monoene production was low, the enzyme activities for the Δ6 16:0-ACP and Δ9 14:0-ACP desaturases were determined (relative to the ubiquitous Δ9 18:0-ACP desaturase) by in vitro assays or by [1-^{14}C]acetate plastid labeling analysis. In vitro acyl-ACP desaturase assays were conducted on homogenates of *Arabidopsis* seeds from lines transformed either with the *T. alata* Δ6 16:0-ACP (D6-24) or the geranium Δ9 14:0-ACP desaturase (9DE-14). As a control, the endogenous Δ9 18:0-ACP desaturase was assayed in the same developing seeds. As shown in Fig. 4, activity of the unusual acyl-ACP desaturases was relatively low for both transgenic lines. In contrast, the activity of the endogenous Δ9 18:0-ACP was beyond the linear range at the earliest time point and thus activity is likely underestimated for this reaction. However, from the 2.5-min time point, it can be

Table 4. Fatty acid profiles of seeds from transgenic *Arabidopsis* lines expressing the Δ4 16:0-ACP desaturase in combination with the coriander seed-specific ACP (Cs-ACP-I)

Genotype[b]	Fatty acid (mol%)			
	Unusual monoenes[c]	Saturates[d]	PUFA + VLCFA[e]	Monoenes[f]
Wild type ($n=6$)	0.0	13.7 ± 0.2	71.0 ± 0.3	15.2 ± 0.4
4DE ($n=10$)	0.6 ± 0.04	13.3 ± 0.2	73.6 ± 0.6	12.5 ± 0.4
4DE/Cs-ACP ($n=18$)	0.7 ± 0.1	13.2 ± 0.1	72.8 ± 0.2	13.2 ± 0.2

[a]Fatty acid profiles of individual genotypes were determined by GC analysis of *n* replicates. Mean values ± SE
[b]Wild-type *Arabidopsis* were transformed with the Δ4 16:0-ACP desaturase (4DE) or co-transformed with 4DE and the coriander seed-specific ACP (Cs-ACP). F_2 seed were analyzed
[c]The sum composition of $16:1^{\Delta4}$ and $18:1^{\Delta6}$
[d]The sum composition of 16:0, 18:0, and 20:0 fatty acids
[e]The sum composition of 18:2, 18:3, and $20:1^{\Delta11}$
[f]The sum composition of $16:1^{\Delta9}$ and $18:1^{\Delta9}$

Table 5. Fatty acid profiles of seeds from transgenic *Arabidopsis* lines expressing the Δ9 14:0-ACP desaturase in combination with *Anabaena* ferredoxin

Genotype[b]	Fatty acid (mol%)			
	Unusual monoenes[c]	Saturates[d]	PUFA/VLCFA[e]	Monoenes[f]
Wild type ($n=1$)	0.0	11.8	73	15.2
AnFd-1 ($n=3$)	0.0	11.1 ± 0.1	71.4 ± 1.3	17.6 ± 1.2
AnFd-2 ($n=3$)	0.0	11.1 ± 0.2	70.1 ± 1.1	17.9 ± 1.7
9DE-14 ($n=3$)	7.4 ± 0.8	12.2 ± 0.1	64.4 ± 0.9	15.9 ± 0.6
9DE-14het ($n=2$)	4.5	12.0	66.8	16.8
9DE-14/AnFd ($n=8$)	3.2 ± 0.1	11.4 ± 0.2	69.6 ± 0.4	15.8 ± 0.2

[a]Fatty acid profiles of individual genotypes were determined by GC analysis of *n* replicates. Mean values ± SE
[b]Wild-type *Arabidopsis* were transformed with the Δ9 16:0-ACP desaturase (9DE-14) or with *Anabaena* ferredoxin (AnFd-1 and AnFd-2). Single-loci, homozygous lines were crossed and F_2 seeds were analyzed. To determine allelic influence on unusual monoene composition, the heterozygous sibling line 9DE-14het was analyzed
[c]The sum composition of $16{:}1^{\Delta 11}$ and $18{:}1^{\Delta 13}$ and $20{:}1^{\Delta 15}$
[d]The sum composition of 16:0, 18:0, and 20:0 fatty acids
[e]The sum composition of 18:2, 18:3, and $20{:}1^{\Delta 11}$ and $22{:}1^{\Delta 13}$
[f]The sum composition of $16{:}1^{\Delta 9}$ and $18{:}1^{\Delta 9}$ and $18{:}1^{\Delta 11}$

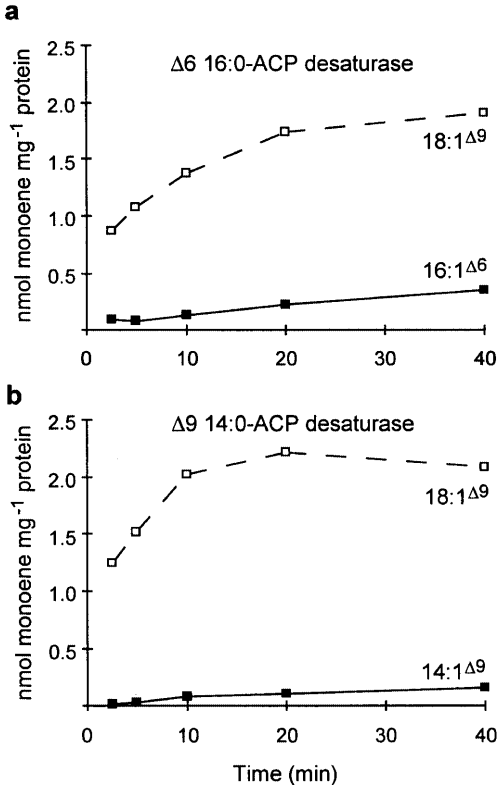

Fig. 4a, b. Acyl-ACP desaturases activities in developing *Arabidopsis* seed. Developing seed were harvested from *Arabidopsis* lines expressing the Δ6 16:0-ACP desaturase (**a**) or the Δ9 14:0-ACP desaturase (**b**) at 8–10 DAF. In vitro desaturase assays containing 30-μg protein extracts were assayed with 16:0-ACP (**a**) or 14:0-ACP (**b**) to compare the activities of the introduced unusual acyl-ACP desaturase (*solid lines*) with the endogenous Δ9 18:0-ACP desaturase (assayed with 18:0-ACP, *dashed lines*)

estimated that activity of the endogenous Δ9 18:0-ACP desaturase is at least 10-fold greater than either introduced unusual acyl-ACP desaturase.

Activity of the unusual acyl-ACP desaturase was also analyzed by [1-^{14}C]acetate labeling of *Arabidopsis* seed plastids isolated from line 9DE-14 (transformed with the geranium Δ9 14:0-ACP desaturase). Consistent with the large differences detected with in vitro desaturase activity, the only unsaturated fatty acid detected after incubation of isolated plastids was $18{:}1^{\Delta 9}$ (data not shown).

In vitro petroselinic acid synthesis is possibly membrane-associated and highly sensitive to disruption

Cahoon and Ohlrogge (1994b) reported that coriander endosperm homogenates actively incorporate [1-^{14}C]malonyl-CoA into petroselinic acid but are inactive in desaturation of acyl-ACP substrates provided to the assays. Although use of coriander ACP and *Anabaena* Fd increases in vitro synthesis of petroselinic acid, oleic acid remains a major product in contrast to its low production in vivo. One hypothesis consistent with these results is that substrate channeling may influence acyl-ACP desaturase activity or that the acyl-ACP desaturase may be part of a multi-component enzyme association. To further explore the involvement of acyl-ACP desaturases in multi-component enzyme associations, in vitro petroselinic acid synthesis was assayed using [1-^{14}C]malonyl-CoA supplied to homogenates or supernatant fractions (after 15,000 *g* centrifugation of homogenates) over a range of protein concentrations. When coriander endosperm homogenates were supplemented with [1-^{14}C]malonyl-CoA and co-factors for fatty acid synthesis, the major labeled product was $18{:}1^{\Delta 6}$ (70–85%) while $18{:}1^{\Delta 9}$ and saturated fatty acids constituted minor products (Fig. 5a). In contrast, when supernatant fractions were assayed, saturated fatty acids were the predominant product while $18{:}1^{\Delta 6}$ was reduced several-fold (Fig. 5a). The influence of centrifugation on acyl-ACP desaturases is particularly evident when comparing activities in the 200-μg protein samples (Fig. 5a, insert). Thus, although acyl-ACP desaturase

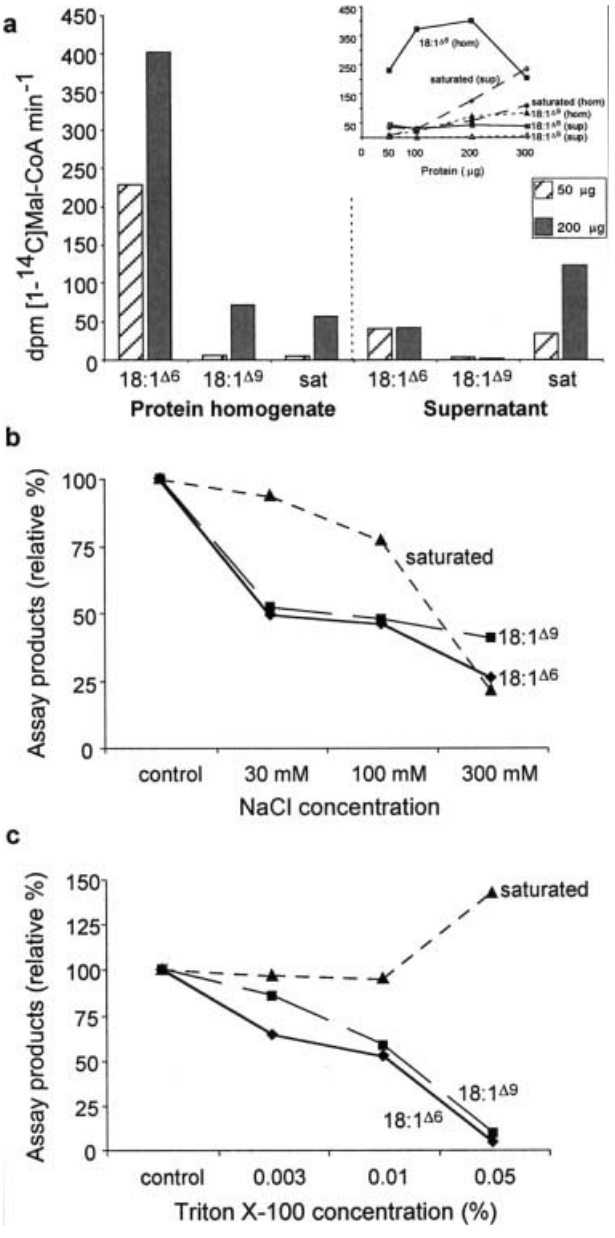

Fig. 5a–c. Petroselinic acid formation by extracts of coriander endosperm. **a** Influence of protein concentration and centrifugation on fatty acid biosynthesis by coriander protein extracts supplied with [1-^{14}C]malonyl-CoA. Production of 18:1$^{\Delta 6}$, 18:1$^{\Delta 9}$ and saturated (*sat*) fatty acids was compared in assays supplied with 50 µg (*hatched bars*) or 200 µg (*filled bars*) of protein extract of endosperm homogenate or 15,000-*g* supernatant. The *insert* shows the effect of increasing protein concentration and centrifugation on production of 18:1$^{\Delta 6}$ (*squares*), 18:1$^{\Delta 9}$ (*triangles*) and saturates (*circles*) in homogenates (*closed symbols*) or supernatant extracts (*open symbols*). **b, c** The influence of salt (**b**) and detergent (**c**) were tested using homogenate protein extracts. Fatty acid products were calculated relative to the control sample. Results represent the average of the two experiments

and fatty acid synthesis enzymes are generally considered to be soluble, centrifugation was detrimental to biosynthesis of 18:1$^{\Delta 6}$. A similar reduction in synthesis of oleic acid to that of petroselinic acid was also observed.

To further evaluate the interaction of acyl-ACP desaturases with additional factors, low concentrations of either NaCl or Triton X-100 were tested using in vitro fatty acid synthesis assays. As shown in Fig. 5b, when the concentration of NaCl was increased from 0 to 300 mM, production of 18:1$^{\Delta 6}$ synthesis was inhibited up to 4-fold. In contrast, production of saturated fatty acid was not substantially reduced until 300 mM NaCl. Similarly, low concentrations of the detergent, Triton X-100 (0.05%) completely inhibited 18:1$^{\Delta 6}$ biosynthesis whereas saturated fatty acid synthesis was unchanged or stimulated (Fig. 5c). Production of 18:1$^{\Delta 9}$ was also inhibited by both treatments.

Discussion

Production of unusual fatty acid structures in transgenic plants by expression of a variety of novel enzymes has been the target of many laboratories. In nearly all cases, production of the targeted fatty acid has been substantially below the high levels found in the native plants. To further the understanding of unusual fatty acid biosynthesis in transgenic plants, we have studied several factors that potentially limit production of unusual monoenoic acids by acyl-ACP desaturases.

In past studies of transgenic plants, transgene copy number and expression levels have been positively, negatively, or indeterminately correlated with the respective phenotype (Hobbs et al. 1993; Matzke and Matzke 1993; Voelker et al. 1996). Voelker et al. (1996) reported that in rapeseed expressing a California Bay 12:0-ACP thioesterase, accumulation of 12:0 correlated positively with enzyme expression level and number of thioesterase gene copies. In our analysis of unusual acyl-ACP desaturases, we found that expression levels of the introduced unusual acyl-ACP desaturases appeared to be higher than that of the endogenous Δ9 18:0-ACP desaturase. However, in contrast to results with lauric acid production, expression levels of the unusual acyl-ACP desaturases were not correlated with the accumulation of unusual monoenoic acids in independent transgenic lines. Thus, it is likely that enzyme expression level is not the major limiting factor in production of unusual monoenes in these transgenic plants.

Production of unusual fatty acids may also be limited by competition with endogenous proteins for substrate or cofactors. For example, when *Lesquerella fenderi* Δ12 hydroxylase is constitutively expressed in *Arabidopsis*, hydroxylated fatty acids are found to account for approximately 10% of seed fatty acids. However, when this Δ12 hydroxylase is expressed in the *fad*2 *Arabidopsis* line (deficient in the highly homologous Δ12 oleate desaturase), hydroxylated fatty acids accumulate to greater than 16% in seeds (Broun et al. 1998). This result could be explained either by reduced competition for cofactors in the *fad*2 mutant or increased 18:1 substrate. To test if reducing competition for cofactors (such as Fd) might

increase unusual acyl-ACP desaturation, we crossed an antisense Δ9 18:0-ACP desaturase line with lines expressing the Δ4 or Δ6 16:0-ACP desaturases. However, this resulted in no appreciable increase in the production of unusual monoenes.

The *Arabidopsis fab1* mutant is deficient in 16:0-ACP elongation and contains higher levels of 16:0 (James and Dooner 1990; Wu et al. 1994). Therefore, *fab1* was transformed with the Δ6 or Δ4 16:0-ACP desaturases. We observed an approximate 3-fold increase in products from the Δ4 16:0-ACP desaturase, but no impact on the products of the Δ6 16:0-ACP desaturase. We do not understand the reasons for this different response, although it may relate to different K_m or other kinetic parameters of the introduced enzymes. Nevertheless, in both cases, despite the increased substrate availability, accumulation of unusual monoenes remained low.

We previously observed that high lauric acid production in transgenic canola induces β-oxidation pathways for lauric acid breakdown (Eccleston and Ohlrogge 1998). Thus, low levels of unusual monoene accumulation could be due to the breakdown of products from the introduced desaturases. Transient accumulation or later losses in unusual monoenoic fatty acids would indicate a possible limitation due to β-oxidation. However, we could find no evidence of a transient accumulation of unusual monoenes that might suggest their breakdown in our transgenic lines (Fig. 2).

Monoene accumulation might also be limited by low rates of incorporation of the fatty acids into TAG. The biosynthesis of TAGs with unusual monoenes in both *T. alata* and coriander exhibits a transient flux of monoenes through phosphatidylcholine prior to incorporation into TAGs (Cahoon and Ohlrogge 1994a; Schultz and Ohlrogge 2000). Furthermore, the specificity of enzymes in the storage-lipid biosynthesis pathway such as acyltransferases (Dutta et al. 1992) may provide barriers that could prevent utilization of unusual monoenes, thus limiting monoene accumulation. However, our results from dual-labeling experiments (Table 1) indicate that incorporation of $18{:}1^{\Delta 6}$ into TAG occurs at rates at least as high as that of $18{:}1^{\Delta 9}$ and therefore we consider this an unlikely limitation to monoene accumulation in these plants.

A further potential limit to acyl-ACP desaturase activity is supply of appropriate co-factors. We previously found specific ACP and Fd isoforms can increase unusual acyl-ACP desaturase activity in vitro by more than 4-fold (Suh et al. 1999; Schultz et al. 2000). We therefore speculated that *Arabidopsis* might not supply ACP or Fd structures optimal for the unusual desaturases and to test this hypothesis, coriander ACP and *Anabaena* Fd were co-expressed with the unusual acyl-ACP desaturases. However, in all cases tested, no substantial increase in unusual monoenes was found with addition of these specialized cofactors.

Although western blots (Figs. 1, 3) indicate the introduced acyl-ACP desaturases are expressed at high levels (relative to the endogenous Δ9 18:0-ACP desaturase), the activity of the introduced unusual acyl-ACP desaturase assayed in homogenates of transgenic seeds was far below the activity of the endogenous Δ9 18:0-ACP desaturase. We do not understand the reasons for the very low activity of the introduced desaturases but it is consistent with the general observations of low specific activity of the unusual acyl-ACP desaturases (Whittle and Shanklin 2001). Western blots of coriander or *T. alata* suggest that the novel acyl-ACP desaturases are expressed at levels similar to the ubiquitous stearoyl-ACP desaturase. However, the production of novel monoenes in coriander or *T. alata* endosperm is up to 10-fold higher than biosynthesis of $18{:}1^{\Delta 9}$ (and its derivatives). Thus, all evidence suggests that the specific activities of the novel desaturases are at least as high in vivo in their native plant as the Δ9 18:0-ACP desaturase. However, when expressed in heterologous systems (either bacterial or transgenic plant), the novel acyl-ACP desaturases have specific activities 10- to 100-fold lower than the Δ9 18:0-ACP desaturase. We have shown that part of this difference may be related to specific isoforms of ACP and/or Fd that are needed for optimal enzyme activity. However, these alone do not seem sufficient to account for the very low activities observed in Fig. 4, or the very low production of monoenes in transgenic plants. One possible interpretation of these results is that the novel desaturases do not fold properly into active enzymes when expressed in non-native environments. Alternatively, it may be that other yet-to-be discovered accessory proteins are needed to provide maximal activity of the acyl-ACP desaturases. The low activity of the novel desaturases in transgenic systems may mean that protein engineering of the stearoyl-ACP desaturase will be a more effective strategy toward production of novel monoenes in oilseed crops (Cahoon et al. 1997).

In this study, we have found the acyl-ACP desaturases are highly sensitive to treatments which disrupt protein–protein interactions (centrifugation, low salt or detergent levels). These results, in addition to our previous work showing $18{:}1^{\Delta 6}$ synthesis in coriander endosperm was sensitive to assay methods (Cahoon and Ohlrogge 1994b) and recent work showing the Δ9 18:0-ACP desaturase is at least partly membrane-associated (Peltier et al. 2000), lead us to speculate acyl-ACP desaturases function as part of a multi-component enzyme association.

Although most enzymes of the plant fatty acid synthesis (FAS) system have been isolated as soluble, non-associated enzyme activities, this may not reflect their in vivo state. Roughan and Ohlrogge (1996) presented evidence indicating FAS may be organized into a multi-enzyme assembly. Recent work in our laboratory has indicated acetyl-CoA carboxylase (which catalyzes the first committed step to FAS) is associated with the plastid membranes (unpublished data). Furthermore, in a recent proteomics-based approach to analyze thylakoid proteins, the Δ9 18:0-ACP desaturase appears to be associated with membranes (Peltier et al. 2000). In addition, the FatB acyl-ACP thioesterase class of enzymes

has a hydrophobic N-terminal domain, which leads to membrane association (J. Froelich and K. Keegstra, Michigan State University, personal communication). Thus, an emerging model for plant fatty acid biosynthesis is a multi-component system that has an association with the plastid membranes. This system may include activities for initiating, elongating, desaturating, and terminating the synthesis of fatty acids. Further progress in understanding unusual acyl-ACP desaturases may therefore lead to a better fundamental understanding of organization and interactions within the entire fatty acid synthesis system.

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